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2. Patent application number *(The Patent Office will fill in this part)* — 9721189.0 — -8 OCT 1997

3. Full name, address and postcode of the or of each applicant *(underline all surnames)*

THE SPEYWOOD LABORATORY LIMITED
14, Kensington Square, London W8 5HH,
United Kingdom. 6765838002
MICROBIOLOGICAL RESEARCH AUTHORITY
Centre for Applied Microbiology & Research,
Porton Down, Salisbury, Wiltshire SP4 0JG,
United Kingdom.

Patents ADP number *(if you know it)* 6516160001

If the applicant is a corporate body, give the country/state of incorporation — United Kingdom

4. Title of the invention

Analgesic Conjugates

5. Full name, address and postcode in the United Kingdom to which all correspondence relating to this form and translation should be sent

Reddie & Grose
16 Theobalds Road
LONDON
WC1X 8PL

Patents ADP number *(if you know it)* — 91001

6. If you are declaring priority from one or more earlier patent applications, give the country and the date of filing of the or of each of these earlier applications and *(if you know it)* the or each application number

Country	Priority application *(If you know it)*	Date of filing *(day/month/year)*

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Number of earlier application	Date of filing *(day/month/year)*

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11. I/We request the grant of a patent on the basis of this application.

Signature: Reddie & Grose — Date: 7 October 1997

12. Name and daytime telephone number of person to contact in the United Kingdom: J M DAVIES 0171-242 0901

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Analgesic Conjugates

Technical field

This invention relates to a class of novel agents that are able to modify nociceptive afferent function. The agents may inhibit the release of neurotransmitters from discrete populations of neurones and thereby reduce or preferably prevent the transmission of afferent pain signals from peripheral to central pain fibres. The agent may be used in or as a pharmaceutical for the treatment of pain, particularly chronic pain.

Background

It is generally accepted that the sensation of pain due to injury or disease is carried from the periphery to the brain by a multi-neuronal pathway. The first part of this system comprises the primary nociceptive afferents which form synapses with secondary neurones in the dorsal horn of the spinal cord, or the nuclei of the cranial nerves. These synapses pass on the incoming information by the release of neurotransmitters and neuromodulators such as glutamate and substance P. These synapses are, therefore, a possible site for interventions designed to alleviate pain, indeed one of the modes of action of the opiate analgesics is to down-modulate neurotransmitter release at these synapses.

Unfortunately, the opiates have a number of limitations as drugs. Firstly, there are a number of chronic pain conditions for which the opiates are not effective. Secondly, the opiates have a number of side effects that are mediated both peripherally (constipation) and centrally (respiratory depression and euphoria) which cause problems for long term use.

There is, therefore, a need for the development of new pharmaceuticals for the treatment of pain, particularly chronic pain.

One approach to this problem is the use of new agents containing fragments of clostridial neurotoxins (WO96/33273).

The clostridial neurotoxins are proteins with molecular masses of the order of 150 kDa. They are produced by various species of bacterium of the genus *Clostridium*, most importantly *C. tetani* and several strains of *C. botulinum*. There are at present

eight different classes of the neurotoxins known: tetanus toxin, and botulinum neurotoxin in its serotypes A, B, C_1, D, E, F and G, and they all share similar structures and modes of action. The clostridial neurotoxins are synthesised by the bacterium as a single polypeptide that is modified post-translationally to form two polypeptide chains joined together by a disulphide bond. The two chains are termed the heavy chain (H), which has a molecular mass of approximately 100 kDa, and the light chain (L), which has a molecular mass of approximately 50 kDa. The clostridial neurotoxins bind to an acceptor site on the cell membrane of the motor neurone at the neuromuscular junction and are internalised by an endocytotic mechanism. The internalised clostridial neurotoxins possess a highly specific zinc-dependent endopeptidase activity which hydrolyses a specific peptide bond in one of three proteins, synaptobrevin, syntaxin or SNAP-25, which are crucial components of the neurosecretory machinery. This activity of the clostridial toxins results in a prolonged muscular paralysis. The zinc-dependent endopeptidase activity of clostridial neurotoxins is found to reside in the L-chain.

Some of the botulinum neurotoxins, most notably serotype A, have been used as pharmaceutical agents for the treatment of focal dystonia. The flaccid paralysing action of the native botulinum toxins makes them appropriate for this use. It has been believed that the clostridial neurotoxins are highly selective for motorneurons due to the specific nature of the acceptor site on those neurones. In various experimental systems, however, it has been shown that the neurotoxins can enter other neuronal cell types, and in all cases the binding activity of clostridial neurotoxins is known to reside in the carboxy-terminal portion of the heavy chain component of the dichain neurotoxin molecule, a region known as H_C.

The use of fragments of clostridial neurotoxins for the desired purpose of analgesia is dependent on the invention of conjugates or derivatives of these molecules that have a specific binding activity that will deliver the L-chain of the neurotoxin to the nociceptive afferent much more efficiently than to other neurones in the relevant anatomical locus. For these agents the delivery includes binding to the cell surface and internalisation via an endosomal compartment leading to entry of the proteolytic activity of the clostridial neurotoxin into the cytosol.

Targeting of extracellular species to specific intracellular locations following endocytosis involves an appreciation of a number of possible targeting strategies. It is understood that early endosomes are one of the key sorting machines of the cell, routing species to late endosome (and onto lysosomes for degradation), recycling to the cell surface or to the Trans-Golgi Network. Intracellular routing determinants

have been suggested that determine the pathway and final destination of particular species (Mellman, 1996, Annu. Rev. Cell Biol., 12, 575-625).

Current data suggests that translocation of neurotoxin occurs from an acidic intracellular compartment, though the exact location and nature of the compartment is unknown (Montecucco & Schiavo, 1994, Mol. Micro., 13, 1-8). In patent application WO96/33273 it is proposed that for an agent to be effective, the agent must target to an appropriate compartment for translocation of the toxin. As an example of specific intracellular targeting, internalisation of the NGF-receptor is by specific endocytosis and retrograde routing (initiated by receptor-ligand complex), via acidic endosomes to the cell body. An agent incorporating NGF is given in support of WO96/33273.

Statement of Invention

The present invention relates to an agent which can reduce and preferably prevent the transmission of pain signals from the periphery to the central nervous system, thereby alleviating the sensation of pain. Specifically, the invention can provide an agent which can reduce and preferably prevent the transmission of pain signals from nociceptive afferents to projection neurones. More specifically, the invention can provide an agent which can inhibit the exocytosis of at least one neurotransmitter or neuromodulator substance from at least one category of nociceptive afferents

In one aspect of the invention, an agent is provided which can be administered into the spinal cord, and which can inhibit the release of at least one neurotransmitter or neuromodulator from the synaptic terminals of nociceptive afferents terminating in that region of the spinal cord.

In a second aspect of the invention, there is provided an agent which can specifically target defined populations of afferent neurones, so that the effect of the agent is limited to that cell type.

In a third aspect of the invention, there is provided a method of treatment of pain which comprises administering an effective dose of the agent according to the invention.

In a fourth aspect of the invention, the agent can be expressed recombinantly as a fusion protein which includes the required components of the agent.

Definitions

Without wishing to be limited by the definitions set down, it is intended in this description that the following terms have the following meanings:

Light chain means the smaller of the two polypeptide chains which form clostridial neurotoxins; it has a molecular mass of approximately 50 kDa and is commonly referred to as L-chain or simply L.

Heavy chain means the larger of the two polypeptide chains which form clostridial neurotoxins; it has a molecular mass of approximately 100 kDa and is commonly referred to as H-chain or simply H.

H_C fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the carboxy-terminal half of the H-chain, or the domain corresponding to that fragment in the intact H-chain. It contains the binding domain of the natural toxin for motor neurones.

H_N fragment means a fragment derived from the H-chain of a clostridial neurotoxin approximately equivalent to the amino-terminal half of the H-chain, or the domain corresponding to that fragment in the intact in the H-chain. It contains a portion of the H-chain which enables translocation of that portion of the neurotoxin molecule such that a functional expression of light chain activity occurs within a target cell. It is the domain responsible, following binding of the molecule to its specific cell surface receptor via a targeting moiety (TM), for translocation of the endopeptidase activity into the cell.

LH_N means a fragment derived from a clostridial neurotoxin that contains the L-chain, or a functional fragment thereof, coupled to the H_N fragment. It can be derived from the intact neurotoxin by proteolysis.

BoNT/A means botulinum neurotoxin serotype A, and is the neurotoxin produced by *Clostridium botulinum* type A; it has a molecular mass of approximately 150kDa.

LH_N/A is LH_N that is derived from *Clostridium botulinum* type A neurotoxin.

Targeting Moiety (TM) means any chemical structure of an agent which functionally interacts with a binding site causing a physical association between the agent and the surface of a primary sensory afferent.

Primary sensory afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system.

Primary nociceptive afferent is a nerve cell that can carry sensory information from the periphery towards the central nervous system, where that information can result in a sensation of pain.

Lectin is any protein that binds to oligosaccharide structures.

Galactose-binding lectin is a lectin that binds to oligosaccharide structures in which the terminal residue is derived from galactose or N-acetylgalactosamine.

Detailed Description of the Invention

It can be seen that an agent for reducing or preventing the transmission of pain signals from peripheral, nociceptive afferent neurones to projection neurones, has many potential applications in the reduction of the sensation of pain, particularly of severe chronic pain.

Lectins are a broadly defined class of proteins, often glycoproteins, that bind to carbohydrate structures. Lectins are found across the whole range of life forms from viruses to mammals. The most commonly exploited sources are the abundant lectins found in the seeds of plants. Lectins have previously been labelled and used as cell surface markers.

According to the invention, there is provided an agent which can inhibit the release of at least one neurotransmitter or neuromodulator or both from the synaptic terminals of nociceptive afferents.

It is known that such an agent can be produced based on the use of fragments of clostridial neurotoxin conjugated to a targeting ligand (WO96/33273). Given the known complexity of intracellular transport and the constraints on construct requirements, it is surprising that conjugates between toxin fragments and a specific selected sub-class of lectins that bind to galactosyl residues (*e.g.* lectins obtained from the genus *Erythrina*) form agents that are particularly potent and selective to produce analgesic agents.

One example of a class of plant-derived, galactose-binding lectins are those that can be purified from the seeds of the genus *Erythrina*. These lectins have been characterised to exist predominantly as non-covalent dimeric proteins with total molecular weights of approximately 60 kDa. Lectins have been isolated from several species including: *E. corallodendron* (Gilboa-Garber and Mizrahi, 1981, Can. J. Biochem. 59, 315-320), *E. cristagalli* (Iglesias et al., 1982, Eur. J. Biochem. 123, 247-252), *E. indica* (Horejsi et al., 1980, Biochim. Biophys. Acta 623, 439-448), *E. arborescens*, *E suberosa*, *E. lithosperma* (Bhattacharyya et al., 1981, Archiv. Biochem. Biophys. 211, 459-470) *E. caffra*, *E. flabelliformis*, *E. latissima*, *E. lysistemon*, *E. humeana*, *E. perrieri*, *E. stricta*, and *E. zeyheri* (Lis et al., 1985, Phytochem. 24, 2803-2809).

These lectins have been analysed for their selectivity for saccharide binding (see e.g. Kaladas et al., 1982, Archiv. Biochem. Biophys. 217, 624-637). They have been found to bind preferentially to oligosaccharides with the a terminal β-D-galactosyl residue.

A second example of a plant-derived, galactose-binding lectin with the desired binding specificity is that obtained from *Glycine max* (soy) beans. This lectin is a tetrameric protein with a total molecular weight of approximately 110 kDa. It binds to oligosaccharides containing galactose or N-acetylgalactosamine residues.

An example of a galactose-binding lectin from bacteria is PA-I, obtained from *Pseudomonas aeruginosa*. PA-I is a D-galactosephilic lectin with a molecular weight of about 13 kDa and it binds to galactose-containing oligosaccharides (Gilboa-Garber and Mizrahi, 1981, Can. J. Biochem. 59, 315-320).

These and other lectins of the sub-class of galactose-binding lectins can be used as targeting moieties (TM) for conjugates of the type described in WO96/33273. The requirements for TMs in these agents are that they show specificity for the primary sensory afferents over other spinal nerves and that they lead to the internalisation of the agents into an appropriate intracellular compartment. The lectins of this invention fulfil these criteria. Surprisingly, in comparison to the TM nerve growth factor (NGF) of WO 96/33273, they can fulfil the second of these criteria more efficiently and can provide agents with better inhibition of afferent neurosecretion.

Thus, in one embodiment of the invention a galactose-binding lectin is conjugated, using linkages that may include one or more spacer regions, to a derivative of the clostridial neurotoxins.

In another embodiment of the invention the agent is expressed in a recombinant form as a fusion protein. The fusion protein is derived from nucleic acid encoding an appropriate fragment of a galactose-binding lectin in addition to any desired spacer domains and nucleic acid encoding all or part of a polypeptide of one serotype of neurotoxin or a chimera derived from the nucleic acid encoding polypeptides from more than one serotype.

In another embodiment of the invention the required LH_N, which may be a hybrid of an L and H_N from different clostridial toxin types, is expressed as a recombinant fusion protein with the galactose-binding lectin, and may also include one or more spacer regions.

In a further embodiment of the invention the required TM and L or LH_N components may be separately expressed in a recombinant form and subsequently linked, covalently or non-covalently, to provide the desired agent.

Exploitation in Industry

The agent described in this invention can be used *in vivo*, either directly or as a pharmaceutically acceptable salt, for treatment of pain.

For example, an agent according to the invention can be administered by spinal injection (epidural or intrathecal) at the level of the spinal segment involved in the innervation of an affected organ for the treatment of pain. This is, for example, applicable in the treatment of deep tissue pain, such as chronic malignant pain.

The present invention will now be described by reference to the following examples together with the Figures which show the following:

Figure 1. SDS-PAGE analysis of fractions from ExL purification
Fractions were subjected to electrophoresis on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie blue. Lane 1 represents molecular weight markers, lanes 2 to 13 are fractions eluted following application of conjugation mixture to the gel filtration column.

Figure 2. Cleavage of SNAP-25 by ExL
ExL was applied to eDRG for 16 hours after which time the hydrophobic proteins were extracted using Triton X-114 and chloroform/methanol precipitation. The extracted protein samples were applied to 4-20% polyacrylamide gradient gels and

subjected to electrophoresis. Proteins were blotted onto nitrocellulose and probed with antibody SMI-81 which recognises BoNT/A cleaved and intact variants of SNAP-25. Lanes 1-3, 4-6, 7-9 and 10-12 represent cells treated with medium, 40μg/ml ExL, 20μg/ml ExL and 40μg/ml LH_N/A respectively.

Figure 3. SDS-PAGE analysis of fractions from EcL purification
Fractions were subjected to electrophoresis on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie blue. Lane 11 represents molecular weight markers. Fractions eluted following application of conjugation mixture to the gel filtration column are shown in lanes 10 to 1, where the first fraction to elute is lane 10. Lane 12 is a sample of the conjugation mixture prior to chromatography.

Example 1. The Production of a conjugate between a lectin from *Erythrina cristagalli* and LH_N/A.

Materials

Lectin from *E. cristagalli* (ExL) was obtained from Sigma Ltd.

LH_N/A was prepared essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* **167**, 175-180.

SPDP was from Pierce Chemical Co.

PD-10 desalting columns were from Pharmacia.

Dimethylsulphoxide (DMSO) was kept anhydrous by storage over a molecular sieve.

Denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using gels and reagents from Novex

Additional reagents were obtained from Sigma Ltd

Methods

The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at -20°C until use.

The ExL was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

The thiopyridone leaving group was removed from the product by reduction with dithiothreitol (DTT; 5 mM; 30 min). The product of this reaction was analyzed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatization achieved. The degree of derivatization achieved was 0.59±0.9 mol/mol. The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

The LH_N/A was desalted into PBSE (PBS containing 1 mM EDTA). The resulting solution (0.5-1.0 mg/ml) was reacted with a four- or five-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 2 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBS.

A portion of the derivatized LH_N/A was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analyzed spectrophotometrically at 280 mm and 343 nm to determine the degree of derivatization. The degree of derivatization achieved was typically 1.5-2.0 mol/mol.

The bulk of the derivatized LH_N/A and the derivatized ExL were mixed in proportions such that the ExL was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for >16 h at 4°C.

The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 50000 molecular weight exclusion limit) before application to a Superose 12 column on an FPLC chromatography system (Pharmacia). The column was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analyzed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie Blue (see Fig. 1). The major band of conjugate has an apparent molecular mass of 150 kDa, this is separated from the bulk of the remaining unconjugated LH_N/A and more completely from the unconjugated ExL. On the gel there are bands due to lectin alone in fractions containing the conjugate, this material is probably due to the non-covalent homo-dimeric nature of the ExL; where only one monomer of ExL is covalently attached to the LH_N/A the other is dissociated from the complex by the SDS in the electrophoretic procedure giving rise to these bands.

The fractions containing conjugate were pooled and stored at 4°C until use.

Example 2. Activities of ExL-LH_N/A in primary cultures of dorsal root ganglion neurons.

Materials

Substance P enzyme linked immunosorbent assay kits were from Cayman Chemical Company.

Western blots reagents were obtained from Novex

Monoclonal antibody SMI-81 was from Sternberger Monoclonals Inc.

Methods

The dorsal root ganglia contain the cell bodies of primary nociceptive afferents. It is well established that in primary *in vitro* cultures of this tissue the neurons retain many of the characteristics of the nociceptive afferents. These characteristics include the ability to release neuropeptides such as substance P in response to chemical stimuli known to cause pain *in vivo* (e.g. capsaicin).

Primary cultures of dorsal root ganglion neurons were established following dissociation of the ganglia dissected from rat embryos (embryological age 12-15 days). The cells were plated into 12 well plates at an initial density of 3×10^5 cells/well in a medium containing NGF (100 ng/ml). After one day in culture, fresh medium containing cytosine arabinoside (10 μM) was added to kill non-neuronal cells. After 2-4 days the cytosine arabinoside was removed. After several more days in culture the medium was replaced with fresh medium containing conjugate or LH_N.

The cells were incubated with these agents for varying times and then the cells were tested for their ability to release the neurotransmitters glutamate and substance P. After the release assays were performed the cells were lysed and the hydrophobic

proteins were extracted by phase partitioning with Triton-X-114 following the method outlined in Boyd, Duggan, Shone and Foster (J. Biol. Chem. **270**, 18216-18218, *1995*).

Substance P release assay

The release of endogenous substance P was effected by collecting cell supernatants after treating the cells for 5 min with either a physiological balanced salt solution or a balanced salt solution in which the potassium ion concentration had been raised to 100 mM with consequent reduction in the sodium ion concentration to maintain isotonicity. Total substance P was measured after extraction in 2 M acetic acid, 0.1% trifluoroacetic acid and subsequent dehydration. Substance P immunoreactivity was measured using an enzyme immunoassay kit (Cayman Chemical Company).

[^{3}H]Glutamate release assay

The release of glutamate was measured after loading the cells with [^{3}H]glutamine as a radiotracer. The [^{3}H]glutamine is converted to [^{3}H]glutamate in the cell, and it is this [^{3}H]glutamate that is taken up by synaptic vesicles and released upon depolarisation of the neuron. The cells are loaded with the [^{3}H]glutamine (5 μCi/ml in HEPES-buffered MEM) for 2 h, then washed twice with HEPES-buffered MEM and thrice with balanced salt solution (BSS). Basal release was assessed with a 3 min incubation with BSS. Stimulated release was determined by a 3 min incubation with BSS in which the potassium concentration had been elevated to 80-100 mM with a consequent reduction in the sodium concentration to maintain isotonicity. All manipulations were performed at 37°C. The cells were lysed by the addition of Triton-X-100 (0.1%, v/v). For the basal and stimulated release superfusates the

glutamate was separated from the glutamine by ion-exchange chromatography over Dowex-1 resin. The relevant fractions were analysed for their ^{3}H content by liquid scintillation counting.

Western blotting

After the determination of substance P or glutamate release the cells were lyzed by the addition of 0.45 ml sodium hydroxide (0.2 M) for 30 min. After this time the solutions were neutralised by the addition of 0.45 ml hydrochloric acid (0.2 M) followed by 0.1 ml of HEPES/NaOH (1 M, pH 7.4). To extract the membrane proteins from these mixtures Triton-X-114 (10%, v/v) was added and incubated at 4°C for 60 min, the insoluble material was removed by centrifugation and the supernatants were then warmed to 37°C for 30 min. The resulting two phases were separated by centrifugation and the upper phase discarded. The proteins in the lower phase were precipitated with chloroform/methanol for analysis by Western blotting.

The samples were separated by SDS-PAGE and transferred to nitrocellulose. Proteolysis of SNAP-25, a crucial component of the neurosecretory process and the substrate for the zinc-dependent endopeptidase activity of BoNT/A, was then detected by probing with an antibody (SMI-81) that recognises both the intact and cleaved forms of SNAP-25 (see Fig. 2).

Example 3. The Production of a conjugate between a lectin from *Erythrina corallodendron* and LH_N/A.

Lectin from *E. corallodendron* (EcL) was obtained from Sigma Ltd.

LH_N/A was prepared essentially by the method of Shone C.C., Hambleton, P., and Melling, J. 1987, *Eur. J. Biochem.* **167**, 175-180.

SPDP was from Pierce Chemical Co.

PD-10 desalting columns were from Pharmacia.

Dimethylsulphoxide (DMSO) was kept anhydrous by storage over molecular sieve.

Denaturing sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed using gels and reagents from Novex.

Additional reagents were obtained from Sigma Ltd.

Methods

The lyophilised lectin was rehydrated in phosphate buffered saline (PBS) to a final concentration of 10 mg/ml. Aliquots of this solution were stored at -20°C until use.

The EcL was reacted with an equal concentration of SPDP by the addition of a 10 mM stock solution of SPDP in DMSO with mixing. After one hour at room temperature the reaction was terminated by desalting into PBS over a PD-10 column.

The thiopyridone leaving group was removed from the product by reduction with dithiothreitol (DTT; 5 mM; 30 min). The product of this reaction was analyzed spectrophotometrically at 280 nm and 343 nm to determine the degree of derivatization achieved. The degree of derivatization achieved was 0.46 ± 0.5 mol/mol. The thiopyridone and DTT were removed by once again desalting into PBS over a PD-10 column.

The LH_N/A was desalted into PBSE (PBS containing 1 mM EDTA). The resulting solution (0.5-1.0 mg/ml) was reacted with a four- or five-fold molar excess of SPDP by addition of a 10 mM stock solution of SPDP in DMSO. After 2 h at room temperature the reaction was terminated by desalting over a PD-10 column into PBS.

A portion of the derivatized LH_N/A was removed from the solution and reduced with DTT (5 mM, 30 min). This sample was analyzed spectrophotometrically at 280 mm and 343 nm in order to determine the degree of derivatization. The degree of derivatization achieved was typically 1.5-2.0 mol/mol.

The bulk of the derivatized LH_N/A and the derivatized EcL were mixed in proportions such that the EcL was in greater than three-fold molar excess. The conjugation reaction was allowed to proceed for >16 h at 4°C.

The product mixture was centrifuged to clear any precipitate that had developed. The supernatant was concentrated by centrifugation through concentrators (with 50000 molecular weight exclusion limit) before application to a Superose 12 column on an FPLC chromatography system (Pharmacia). The column was eluted with PBS and the elution profile followed at 280 nm.

Fractions were analyzed by SDS-PAGE on 4-20% polyacrylamide gradient gels, followed by staining with Coomassie Blue (see Fig. 3). The major band of conjugate has an apparent molecular mass of 150 kDa, this is separated from the bulk of the remaining unconjugated LH_N/A and more completely from the unconjugated EcL. On the gel there are bands due to lectin alone in fractions containing the conjugate, this material is probably due to the non-covalent homo-dimeric nature of the EcL; where only one monomer of EcL is covalently attached to the LH_N/A the other is

dissociated from the complex by the SDS in the electrophoretic procedure giving rise to these bands.

The fractions containing conjugate were pooled and stored at 4°C until use.

Claims

1. An agent for the treatment of pain that comprises a galactose-binding lectin linked to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises (i) the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to (ii) a part of the heavy (H) chain with membrane translocating activity.

2. An agent according to Claim 1 in which the lectin binds to oligosaccharides that contain terminal β-D-galactosyl residues

3. An agent according to Claim 1 in which the lectin binds to oligosaccharides that contain terminal α-D-galactosyl residues

4. An agent according to Claim 1 in which the lectin binds to oligosaccharides that contain N-acetylgalactosamine

5. An agent according to any previous Claim in which the lectin is derived from a species of plant.

6. An agent according to Claim 5 in which the lectin is derived from a species of the genus *Erythrina*.

7. An agent according to Claim 6 in which the lectin is derived from *E. cristagalli*.

8. An agent according to Claim 6 in which the lectin is derived from *E. corallodendron*.

9. An agent according to Claim 6 in which the lectin is derived from *E. indica*.

10. An agent according to Claim 6 in which the lectin is derived from *E. arborescens*.

11. An agent according to Claim 6 in which the lectin is derived from *E. suberosa*.

12. An agent according to Claim 6 in which the lectin is derived from *E. lithosperma*.

13. An agent according to Claim 6 in which the lectin is derived from *E. caffra*.

14. An agent according to Claim 6 in which the lectin is derived from *E. flabelliformis*.

15. An agent according to Claim 6 in which the lectin is derived from *E. latissima*.

16. An agent according to Claim 6 in which the lectin is derived from *E. lysistemon*.

17. An agent according to Claim 6 in which the lectin is derived from *E. humeana*.

18. An agent according to Claim 6 in which the lectin is derived from *E. perrieri*.

19. An agent according to Claim 6 in which the lectin is derived from *E. stricta*.

20. An agent according to Claim 6 in which the lectin is derived from *E. zeyheri*.

21. An agent according to Claims 1-5 in which the lectin is derived from *Glycine max*.

22. An agent according to Claims 1-5 in which the lectin is derived from *Arachis hypogaea*.

23. An agent according to Claims 1-5 in which the lectin is derived from *Bandeirea simplicifolia*.

24. An agent according to Claim 1-4 in which the lectin is of mammalian origin.

25. An agent according to Claim 1-4 in which the lectin is derived from bacteria.

26. An agent according to Claim 25 in which the lectin is derived from *Pseudomonas aeruginosa*.

27. An agent according to any preceding Claim in which the lectin has been produced using recombinant technology.

28. An agent according to Claims 1-27 in which the lectin has been modified by proteolysis.

29. An agent according to Claims 1-27 in which the lectin has been chemically modified.

30. An agent according to any of Claims 1-29 which comprises the lectin coupled to a clostridial neurotoxin in which the H_C domain of the H-chain is removed or modified.

31. An agent according to any of Claims 1-30 in which the H-chain is modified by chemical derivatisation to reduce or remove its native binding affinity for motor neurons.

32. An agent according to any of Claims 1-30 in which the H-chain is modified by mutation to reduce or remove its native binding affinity for motor neurons.

33. An agent according to any of Claims 1-30 in which the H-chain is modified by proteolysis.

34. An agent according to Claim 30 in which the H_C domain is completely removed leaving only the H_N-fragment of a clostridial neurotoxin.

35. An agent according to any preceding Claim in which the clostridial neurotoxin component is obtained from botulinum neurotoxin.

36. An agent according to any preceding Claim in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type A.

37. An agent according to Claims 1-35 in which the clostridial neurotoxin component is obtained from botulinum neurotoxin type B.

38. An agent according to any of Claims 1-35 which is formed by the coupling of a galactose-binding lectin to the LH_N fragment of botulinum neurotoxin type A.

39. An agent according to Claim 38 which is formed by the coupling of the galactose-binding lectin from *Erythrina cristagalli* to the LH_N fragment of botulinum neurotoxin type A.

40. An agent according to Claim 38 which is formed by the coupling of the galactose-binding lectin from *Erythrina corallodendron* to the LH_N fragment of botulinum neurotoxin type A.

41. An agent according to Claim 38 which is formed by the coupling of the galactose-binding lectin from *Glycine max* to the LH_N fragment of botulinum neurotoxin type A.

42. An agent according to any of Claims 1-41 in which the H-chain is obtained from a different clostridial neurotoxin than that from which the L-chain is obtained.

43. An agent according to Claim 42 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from botulinum neurotoxin type B.

44. An agent according to Claim 42 in which the H-chain is obtained from botulinum neurotoxin type A and the L-chain from tetanus neurotoxin.

45. An agent according to Claim 43 which comprises a galactose-binding lectin linked to the H_N fragment of botulinum neurotoxin type A and the L-chain of botulinum neurotoxin type B.

46. An agent according to Claim 45 which comprises a galactose-binding lectin linked to the H_N fragment of botulinum neurotoxin type A and the L-chain of tetanus neurotoxin.

47. An agent according to any preceding Claim in which the L-chain or L-chain fragment is linked to the H-chain by a direct covalent linkage.

48. An agent according to any of Claims 1-46 in which the L-chain or L-chain fragment is linked to the H-chain by a covalent linkage which includes one or more spacer regions.

49. An agent according to any preceding Claim in which the clostridial neurotoxin derivative incorporates polypeptides produced by recombinant technology.

50. An agent according to any preceding Claim in which the lectin is linked to the clostridial neurotoxin-derived component by a direct covalent linkage.

51. An agent according to any of Claims 1-49 in which the lectin is linked to the clostridial neurotoxin-derived component by a covalent linkage which includes one or more spacer regions.

52. An agent according to any preceding Claim in which the lectin and clostridial neurotoxin components are produced as a recombinant fusion protein.

53. An agent according to any preceding Claim in which the lectin protein has been modified from its native polypeptide sequence whilst retaining an ability for the protein to bind to oligosaccharide structures, in which the terminal residue is derived from galactose or N-acetylgalactosamine.

54. An agent according to Claim 53 in which the protein modification results from modification of the nucleic acid coding for the lectin protein from its native sequence.

55. An agent according to any preceding Claim which prevents the release of a neurotransmitter or neuromodulator from a primary sensory afferent.

56. An agent according to any preceding Claim which inhibits the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent.

57. A method for obtaining an agent according to any preceding Claim which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin, in which the derivative of the clostridial neurotoxin comprises (i) the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to (ii) a part of the heavy (H) chain with membrane translocating activity.

58. A method for obtaining an agent according to any of Claims 1-56 which comprises the covalent attachment of a galactose-binding lectin to a derivative of a clostridial neurotoxin with the inclusion of one or more spacer regions, in which the derivative of the clostridial neurotoxin comprises (i) the L-chain or an L-chain fragment which includes the active proteolytic enzyme domain of the light (L) chain, linked to (ii) a part of the heavy (H) chain with membrane translocating activity.

59. A method for obtaining an agent according to any of Claims 1-56 which comprises constructing a genetic construct which codes for the agent, incorporating said construct into a host organism and expressing the construct to produce the agent.

60. A method of controlling the release of a neurotransmitter or neuromodulator from a primary sensory afferent by applying the agent of any one of Claims 1-56.

61. A method of controlling the release of a neurotransmitter or neuromodulator from a primary nociceptive afferent by applying the agent of any one of Claims 1-56.

62. A method of controlling the transmission of sensory information from a primary sensory afferent to a projection neuron by applying the agent of any one of Claims 1-56.

63. A method of controlling the transmission of sensory information from a primary nociceptive afferent to a projection neuron by applying the agent of any one of Claims 1-56.

64. A method of controlling the sensation of pain by applying the agent of any one of Claims 1-56.

65. Use of the agent according to any one of Claims 1-56 or a pharmaceutically acceptable salt thereof as a medicament for the alleviation of pain.

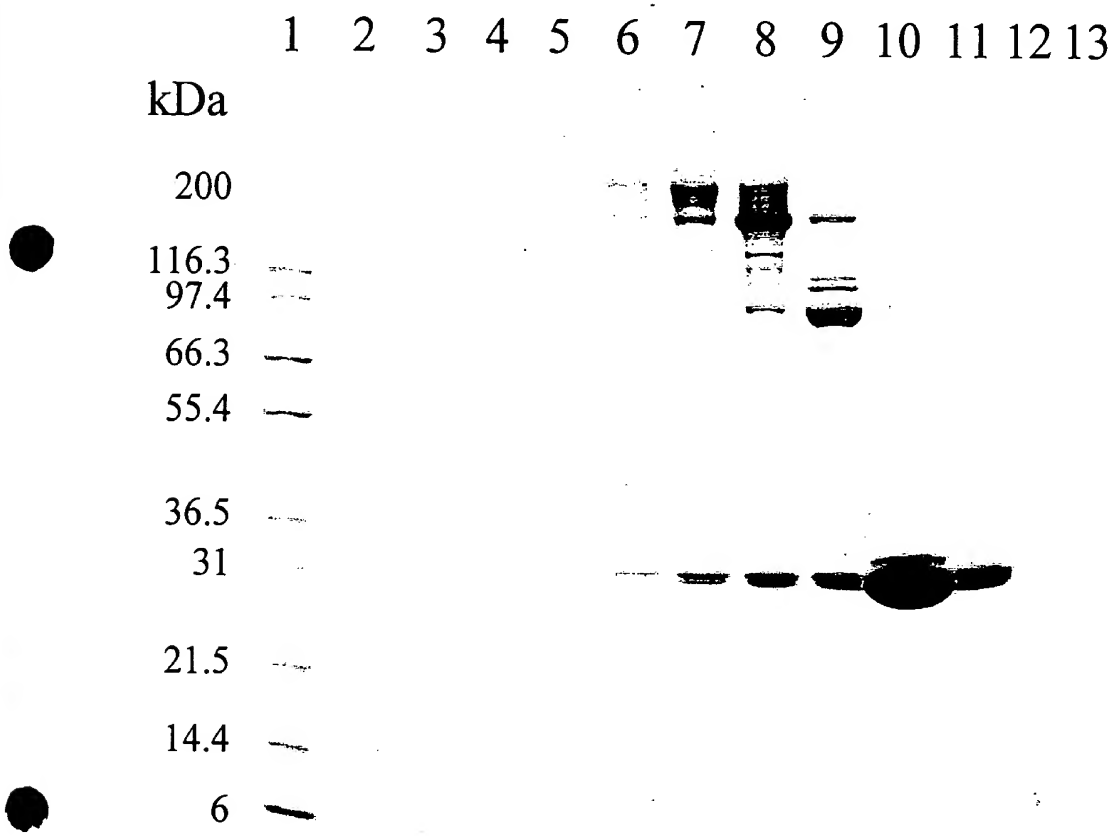
66. Use of the agent according to any one of Claims 1-56 or a pharmaceutically acceptable salt thereof as a medicament for the prevention of pain.

67. Use of the agent according to any one of Claims 1-56 in the manufacture of a medicament for the alleviation of pain.

68. Use of the agent according to any one of Claims 1-56 in the manufacture of a medicament for the prevention of pain.

69. A method of alleviating pain which comprises administering an effective dose of the agent according to any one of Claims 1-56.

70. A method of preventing pain which comprises administering an effective dose of the agent according to any one of Claims 1-56.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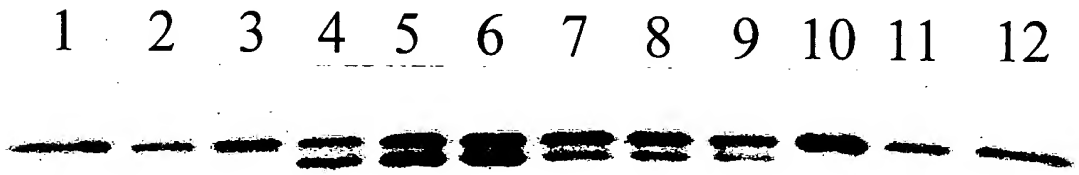
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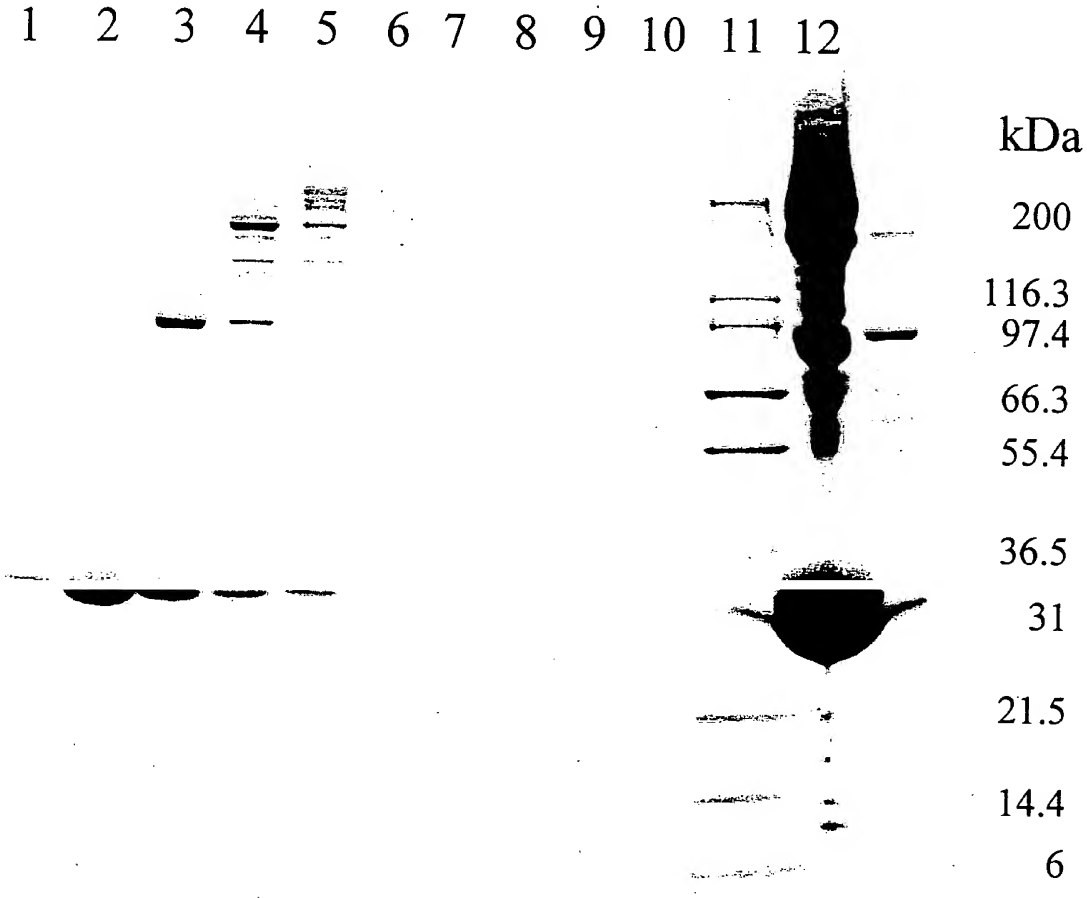
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